Induction of Micronuclei in Human TK6 Lymphoblasts by 2-Dodecylcyclobutanone, a Unique Radiolytic Product of Palmitic Acid

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ABSTRACT: Palmitic acid, the most abundant fatty acid in the human diet, causes oxidative DNA damage, DNA strand breakage, necrosis, and apoptosis in human and rodent cells *in vitro*, while excess fatty acids in the diet increase the risk of colon cancer *in vivo*. Irradiation of palmitic acid leads to the formation of 2-dodecylcyclobutanone (2-DCB), a unique radiolytic product formed at part-per-million levels in fats. Recent research has raised the possibility that, like palmitic acid, 2-DCB can also cause DNA strand breaks in human and rodent cells. In order to more fully understand the clastogenic potential of 2-DCB, it was tested for the ability to induce the formation of micronuclei (MN) in human TK6 lymphoblasts. TK6 lymphoblasts were exposed to 13.3, 26.5, and 53 μM of 2-DCB for 24 h, both with and without exogenous metabolic activation (EMA). The number of MN per 1000 binucleated cells was induced 2.65-fold without EMA, and 2.85-fold in the presence of EMA at the highest 2-DCB concentration of 53 μM. Cytotoxicity of the TK6 lymphoblasts treated with 53 μM 2-DCB was 51.7% in the absence of EMA, and 61.7% in the presence of EMA. Thus, like palmitic acid, 2-DCB may be weakly clastogenic against human cells *in vitro*. Given the amount of 2-DCB that would be consumed when compared with that of its clastogenic parent fatty acid, 2-DCB is unlikely to have any significant impact on human health.

Keywords: cancer, clastogen, 2-dodecylcyclobutanone, palmitic acid, radiation

Introduction

Radiation pasteurization of meat and poultry inactivates pathogenic microorganisms such as *Escherichia coli* O157, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, and *Yersinia enterocolitica*, and has the potential to significantly reduce the incidence of hospitalizations and deaths linked to consumption of food contaminated with those food-borne pathogens (Tauxe 2001). Long-term feeding studies conducted in multiple animal species that used large quantities of radiation-pasteurized and sterilized meat and poultry (6 to 74 kGy) have found no increased risk of cancer or birth defects (WHO 1994; FDA 2005). Despite this fact, "consumer groups" opposed to food irradiation and other modern food safety technologies and agricultural practices have expressed concern that unique radiolytic products formed in foods by ionizing radiation could be genotoxic, and therefore that irradiated foods are carcinogenic (Anonymous 2003; WHO 1994; FDA 2005). This opposition to food irradiation has grown more vocal since irradiated ground beef was made available, on a voluntary basis, as part of the USDA's National School Lunch Program (USDA 2003).

Exposure of foods containing fatty acids, such as meat and poultry, to ionizing radiation leads to the formation of compounds called 2-alkylcyclobutanones (2-ACBs), which are not detectable in nonirradiated meat products (LeTellier and Nawar 1972; Boyd and others 1991). The most abundant fatty acid in meat is palmitic acid, and the most abundant 2-ACB formed in meat is 2-dodecylcyclobutanone (2-DCB) (Crone and others 1992a, 1992b). Cleavage of the acyl-oxygen bond of palmitic acid by ionizing radiation leads to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid, with an alkyl group in the second ring position, or 2-DCB ($C_{16}H_{30}O$; FW 238.41) (LeTellier and Nawar 1972) (Figure 1). A person consuming an irradiated, and then cooked, ground beef patty (100 to 125 g) would be expected to consume approximately 3.3 to 6.0 μg of 2-DCB, which would be 120 to 240 ng/kg/bw for a 25-kg person, 60 to 120 ng/kg/bw for a 50-kg person, or 30 to 60 ng/kg/bw for a 100-kg person (Knoll and others 2006; calculated from Anonymous 2003).

The most effective way to evaluate food-processing technologies for safety is to conduct long-term feeding studies in animals, because of the complex chemical changes that often occur in food as a result of exposure to processing technologies (FDA 2005). However, because the concentration of 2-DCB that would be consumed is above the 1.5-μg/d limit that would require its safety testing as an indirect food additive (FDA 2000), "consumer groups" have requested that 2-DCB and other 2-ACBs that might exceed the 1.5-μg/d limit be tested for genotoxicity. As a result of these requests, multiple laboratories have tested purified 2-DCB in mutagenicity assays, including the *Salmonella* Mutagenicity Test, the *E. coli* TRP Assay, the 5-Fluorouracil Mutagenesis Assay, and the Mouse Lymphoma Assay, and have failed to detect a 2-DCB-induced increase in mutations (Burnouf and others 2002; Sommers 2003; Gadgil and Smith 2004; Sommers and Schiestl 2004; Sommers and Mackay 2005; Sommers 2006). In addition, Gadgil and Smith (2004) found that 2-DCB was no more cytotoxic *in vitro* than commonly used GRAS food additives, using the MicroTox Test.

In contrast to mutagenicity tests, 2-DCB has produced equivocal results in DNA fragmentation assays, which often occurs as a result of

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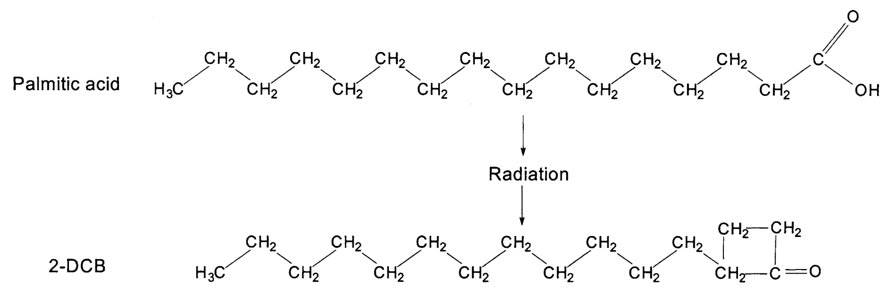

Figure 1 — Structure of palmitic acid and 2-dodecylcyclobutanone (2-DCB) as determined by LeTellier and Newar (1972)

oxidative damage to DNA. When 2-DCB was tested for the ability to induce DNA strand breaks in human and rodent colon tumor cells *in vitro* using the Comet Assay, no increase in DNA strand breakage was observed (Burnouf and others 2002). However, in the same study, 2-DCB-induced oxidative DNA damage was detected in using an alkaline DNA unwinding assay commonly used in the study of DNA repair. Sommers and Schiestl (2004) tested 2-DCB for the ability to induce intrachromosomal recombination using the yeast DEL Assay, which has been used for many years to identify compounds that produce oxidative damage to DNA, but obtained negative results at concentrations up to 5 mg/mL. In an *in vivo* study in which rodents were fed 2-DCB by gavage, a 2-DCB dose of 1.12 mg/kg/bw did not induce DNA strand breaks in colon cells, but a 2-DCB dose of 14.9 mg/kg/bw induced a 2- to 3-fold increase in DNA strand breakage as detected by the Comet Assay (Delincée and others 1999). More recently, Knoll and others (2006) detected a 2- to 3-fold increase in DNA strand breakage in human cells treated with 2-DCB using the Comet Assay, and noted a similar increase in chromosomal rearrangements using the 24-Color Fluorescent *In Situ* Hybridization (FISH) Assay. The equivocal nature of responses in the various studies could be due to the use of different cell lines and organisms, the variability of exposure times to the test compound, and the different range of 2-DCB concentrations.

The Micronucleus Assay has been used for decades to determine a test compound's ability to cause chromosome fragmentation, or clastogenicity, which leads to the formation of "micronuclei (MN)," or chromatin-containing bodies that consist of chromosome fragments, and sometimes whole chromosomes, within cells that can then be detected by fluorescence microscopy (Fenech and Morley 1985) (Figure 2). In this study, in order to further evaluate the potential clastogenicity of 2-DCB, it was tested for the ability to induce the formation of MN in human TK6 lympholasts *in vitro*, both with and without exogenous metabolic activation (EMA).

Materials and Methods

Cell and growth medium

The human lymphoblastoid cell line TK6 (American Type Culture Collection (ATCC), Manassas, Va., U.S.A.) was used in this study. Cells were maintained as exponentially growing cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum (ATCC), penicillin (100 U/mL), and streptomycin (100 U/mL) (ATCC). The Dulbelco's phosphate buffered saline that was used for washing cells was also obtained from ATCC. Cells were maintained in a humidified incubator (37 °C, 5% CO_2) during the course of the study.

Chemicals and reagents

Methyl methanesulfonate (MMS), cytochalasin B, acridine orange, dimethylsulfoxide (DMSO), and ethanol were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.). S9 fraction from Aroclor 1254-induced male Sprague-Dawley rats and associated buffers as well as the positive control compound 2-aminoanthracene (2-AA) were obtained from Moltox, Inc. (Boone, N.C., U.S.A.). The test compound 2-DCB (CAS# 35493-46-0) was obtained from the Fluka subsidiary of Sigma-Aldrich Chemical Co.

Assay procedure

The procedure as described by the Linus Pauling Institute (2005) was used in this study. TK6 lymphoblasts were adjusted to a density of 10^5 cells/mL, the media supplemented with cytochalasin B (3 μg/mL), and aliquoted (2.0 mL) into sterile 12-well microtiter plates. Ethanol (1%) was used as the solvent control for untreated cells, and 2-DCB was dissolved in ethanol and added to the cells to final concentrations of 13.3, 26.5, and 53 μM with a final ethanol concentration of 1%. DMSO (final concentration 0.5%) was used as the solvent for the positive control compounds MMS (13 μg/mL) in the absence of S9 fraction, or 2-AA (0.1 μg/mL) in the presence of 1%

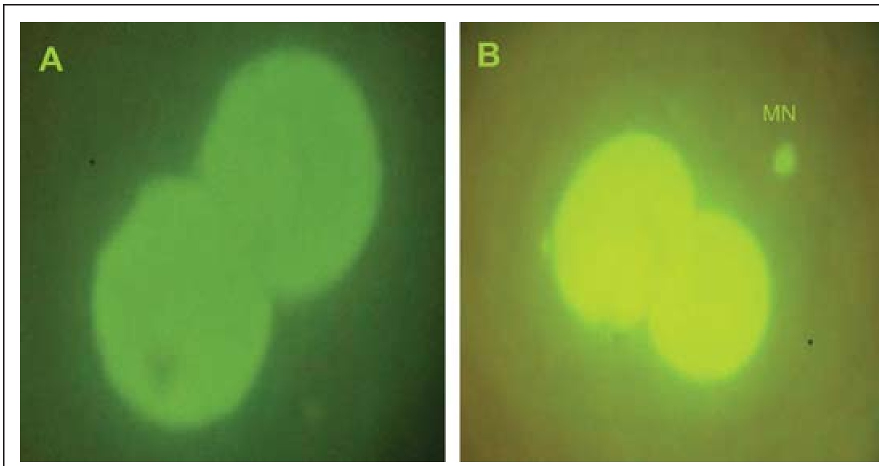

Figure 2 — Binucleated TK6 lymphocyte (A) without micronuclei and (B) with micronuclei

S9 fraction. Cells were incubated in the presence of the chemicals for 24 h, pelleted by centrifugation, washed once with phosphate buffered saline, and fixed in a solution of methanol and acetic acid (20:1). The fixed cells were then transferred to microscope slides, allowed to air-dry, stained with acridine orange (10 μg/mL) in phosphate buffered saline, destained with phosphate buffered saline, and examined for the presence of MN, using an Axiolab HB50 fluorescence microscope at 40× magnification.

Only binucleated (BN) cells were scored for the presence of MN, using the criterion outlined by Fenech and Morley (1985). Approximately 1000 BN cells were scored per test compound concentration per experiment. Each experiment was conducted independently 3 times. Cytokinesis-Block Proliferative Index (CBPI), a measure of the effectiveness of the cell cycle block, is defined as (Number of mononucleated cells) + (2 × Number of binucleated cells) + (3 × Number of multinucleate cells)/100. Cytotoxicity was calculated as $100 - 100[(CBPI_T - 1)/(CBPI_C - 1)]$, where T is the treated culture and C is the control culture (Fenech and Morley 1985; OECD 1997).

Statistical analysis

Each experiment was conducted independently 3 times, with 1000 BN cells counted at each 2-DCB concentration in each experiment ($n = 3$). For determination of cytotoxicity, 100 cells were counted per test compound concentration per experiment ($n = 3$). Statistically significant differences ($n = 3, \alpha = 0.05$) were determined by Student's *t* test, using the Statistical Analysis ToolPak of Microsoft Excel™ Software (Microsoft Corp., Redmond, Wash., U.S.A.).

Results and Discussion

Results of the Micronucleus Assay are listed in Table 1 and are presented as the number of cells with MN per 1000 BN cells. The number of MN/1000 BN cells in the control samples is consistent with published literature, and no increase in MN/1000 BN cells was observed in experiments with and without EMA at 2-DCB concentrations of 13.3 or 26.5 μM. However, a statistically significant increase in MN, single MN per cell, was observed at the 53.0 μM concentration, with a 2.67-fold increase observed in MN/1000 BN cells without EMA, and a 2.85-fold increase in the presence of EMA. The increase in the number of MN per 1000 BN cells at the 53 μM concentration was consistent in the 3 independent experiments that were performed. The CBPI, which is a measure of the effectiveness of the cell cycle block by cytochalasin B, was in the expected range, and the cytotoxicity of the TK6 lymphoblasts exposed to 2-DCB was 51.7% without EMA and 55.3% with EMA, which are close to the maximum recommended level of 60% cytotoxicity for the assay.

Microscopic examination of the TK6 lymphoblasts prior to fixation revealed a necrotic appearance of the cells at the 53-μM concentration, and the TK6 cells were completely lysed at a 2-DCB concentration of 106 μM. In a previous study, Sommers (2006) found that 2-DCB-treated TK6 lymphoblasts were necrotic and sensitive to centrifugation, an indicator of cell membrane disruption. Despite the fact that no dose–response was obtained, the increase in formation of MN observed in this study is consistent with an increase in DNA strand breaks or chromosomal rearrangements observed in other studies. Knoll and others (2006) found that 2-DCB (150 μM, 6 h exposure) increased the frequency of chromosomal aberrations, including deletions, isochromosomes, and translocations, approximately 3-fold in LT97 adenoma cells *in vitro*, using the 24-Color FISH Assay. In the same study, a 2- to 3-fold increase in DNA strand breakage, using the Comet Assay, was observed in human primary colon cells obtained from patients who had surgery for polyps, diverticulitis, and colon cancer, and in LT97 adenoma cells exposed to 150 μM 2-DCB for 30 to 60 min, but not in the HT29clone19A colonic cancer cell line. In an earlier *in vivo* study conducted in rats, colon cells isolated from the animals following an exposure of 14.9 mg/kg/bw, but not 1.12 mg/kg/bw, showed a doubling of DNA strand breakage, using the Comet Assay. Using both human and rodent colon tumor cells *in vitro*, Burnouf and others (2002) reported no increase in tail length or intensity following exposure of 150 μM 2-DCB for 30 min, using the Comet Assay. Thus, results from multiple studies using tumor cells and primary cells may place 2-DCB in the "weak" range as a potential clastogen, because a 0- to 3-fold increase in DNA strand breaks, chromosomal aberrations, or MN has been observed.

The clastogen palmitic acid comprises approximately 25% of the fatty acids in beef tallow, or gram quantities in a typical serving of ground beef (FDA 2005). However, a person would be expected to consume approximately 3 to 6 μg of 2-DCB, derived from palmitic acid, in a 100 to 125 g serving of cooked, irradiated ground beef (Knoll and others 2006; calculated from Anonymous 2003). The weak clastogenic activity of 2-DCB in this study is not unexpected. The parent molecule of 2-DCB, palmitic acid, has previously been shown to be a strong inducer of oxidative DNA damage, DNA strand breaks, cell membrane damage, necrosis, and apoptosis in human and rodent cells *in vitro* at concentrations ranging from 50 to 200 μM (Beeharry and others 2003; de Sousa Andrade and others 2005). Other fatty acids that cause cell membrane damage and chromosome fragmentation in human and rodent cells *in vitro* include linoleic acid (100 μM), aracidonic acid (100 μM), and thermally oxidized dietary oils (Udilova and others 2003; de Sousa Andrade and others 2005). Thus, the effects of 150 μM 2-DCB on human primary and adenoma cells noted by Knoll and others (2006), and the effect of 53 μM 2-DCB on the TK6 lymphoblasts used in this study, are similar to those caused by palmitic acid and other fatty acids.

Excessive consumption of saturated fatty acids is associated with tumor promotion in the colons of rodents, and an increased risk of colon cancer in humans (Weisburger 1997; Yang and others 1998; Bartsch and others 1999; Zock 2001). Fatty acids and oxidized lipids, commonly found in raw and cooked meats and poultry, are known to be mutagenic (Scheutwinkel-Reich and others 1980; Spigarn and

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Table 1 — Induction of micronuclei in human TK6 lymphoblasts by 2-dodecylcyclobutanone

	No S9 fraction			1% S9 fraction		
Conc. (μM)	Micronuclei (per 1000 BN)	CBPI	Cytotoxicity	Micronuclei (per 1000 BN)	CBPI	Cytotoxicity
0	16.7 ± 3.18	2.32 ± 0.04	0.00 ± 0.00	22.0 ± 2.65	1.70 ± 0.79	0.00 ± 0.00
13.3	12.7 ± 2.67	1.17 ± 0.10	12.2 ± 4.34	22.3 ± 2.85	1.53 ± 0.71	12.9 ± 5.28
26.5	13.7 ± 0.88	1.80 ± 0.10	39.7 ± 6.50	25.7 ± 1.88	1.43 ± 0.67	20.9 ± 11.3
53.0	44.7 ± 2.03*	1.64 ± 0.09	51.7 ± 5.20	62.7 ± 2.73*	1.09 ± 0.51	60.7 ± 4.54
Pos. control	95.0 ± 6.07	1.86 ± 0.10	25.8 ± 5.83	103.0 ± 10.9	1.55 ± 0.09	55.3 ± 5.41

Each experiment was conducted independently 3 times. Standard errors (SEM) are shown in parenthesis.
*Statistically significant increases in micronucleus formation were determined using Student's *t* test.
CBPI = the Cytokinesis-Block Proliferative Index, is a measure of the effectiveness of the cycle block.

others 1981; Vithayathil and others 1983; Viksi and Joner 1993), cause oxidative damage to DNA, and induce cell proliferation both *in vitro* and *in vivo* (Bartsch and others 1999; Coquhoun and Schumacher 2001;). Palmitic acid induces DNA strand breakage in human and rodent cells (Beeharry and others 2003; de Sousa Andrade and others 2005). In contrast, 2-DCB is a noninducer, or a weak inducer, of DNA strand breaks in yeast, rodent, and human cells. It appears that 2-DCB may in fact be less genotoxic than its parent fatty acid. Future research on 2-DCB clastogenicity should include direct comparisons between 2-DCB and palmitic acid to clarify this possibility.

The results presented herein, when viewed in combination with the long-term studies conducted in multiple animal species, which failed to find adverse effects associated with consumption of irradiated meat and poultry, support the position of the FDA and WHO (WHO 1994; FDA 2005) regarding the safety and wholesomeness of irradiated foods.

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